EXHIBIT A

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Inhibition of Tumor Metastasis by Heparanase Inhibiting Species of Heparin

Abstract

Heparanase activity correlates with the metastatic potential of lymphoma, melanoma and mammary adenocarcinoma cell lines. We investigated the ability of various modified species of heparin and size-homogeneous oligosaccharides derived from depolymerized heparin to inhibit (1) heparanase-mediated degradation of heparan sulfate in a naturally produced subendothelial extracellular matrix (ECM), and (2) lung colonization of B16-BL6 melanoma cells in C57BL mice. Inhibition of heparanase was best achieved by heparin species containing 16 or more sugar units and having sulfate groups at both the N and O positions. Low-sulfate oligosaccharides were less effective heparanase inhibitors than medium- and high-sulfate fractions of the same-size saccharide. While O-desulfation abolished the heparanase-inhibiting effect of heparin, O-sulfated, N-substituted (e.g. N-acetyl or N-hexanoyl) species of heparin retained high inhibitory activity. Potent inhibitors of heparanase activity were also efficient inhibitors of tumor invasion and lung colonization. Heparin fractions with high and low anticoagulant activity expressed similar high antiheparanase and antimetastatic activities. Structural requirement for the inhibition of melanoma cell heparanase and lung colonization by species of heparin were different from those identified for (1) release of ECM-bound basic fibroblast growth factor (b-FGF) and (2) stimulation of b-FGF receptor binding and mitogenic activity. These results indicate that various nonanticoagulant species of heparin and other polyanionic molecules differing in size, sulfation and substituted groups can be designed to elicit specific effects resulting in the inhibition of cell invasion in tumor metastasis and autoimmunity, or stimulation of neovascularization and wound healing.

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Circulating tumor cells arrested in the capillary beds of different organs must invade the endothelial cell lining and degrade its underlying basement membrane in order to escape into the extravascular tissue(s) where they establish metastasis [1, 2]. Metastatic tumor cells often attach at or near the intercellular junctions between adjacent endothelial cells followed by rupture of the junctions, retraction of the endothelial cell borders and migration through the breach in the endothelium toward the exposed underlying basal lamina [1, 2]. Once enveloped between endothelial cells and the basal lamina, the invading cells must degrade the subendothelial glycoproteins and proteoglycans in order to migrate out of the vascular compartment. Our studies on the extravasation of normal and malignant blood-borne cells focus on the ability of cells to degrade heparan sulfate (HS) proteoglycans (HSPG) in a basement-membrane-like extracellular matrix (ECM) produced by cultured corneal and vascular endothelial cells [3, 4]. This ECM closely resembles the in vivo subendothelium in its morphological appearance and molecular composition. It contains primarily collagens (mostly types III and IV, with smaller amounts of types I and V), proteoglycans (mostly HSPGs and dermatan sulfate proteoglycans, with smaller amounts of chondroitin sulfate proteoglycans), laminin, entactin, fibronectin and elastin [5].

HSPGs are ubiquitous macromolecules associated with the cell surface and ECM of a wide range of cells of vertebrate and invertebrate tissues [6, 7]. The basic HSPG structure consists of a protein core to which several linear heparan sulfate chains are covalently attached. The polysaccharide chains are typically composed of repeating hexuronic and D-glucosamine disaccharide units that are substituted to a varying extent with N- and O-linked sulfate moieties and N-linked acetyl groups [6, 7]. Studies on the involvement of ECM molecules in cell attachment, growth and differentiation have revealed a central role for HSPGs in embryonic morphogenesis, angiogenesis, neurite outgrowth and tissue repair [6–8]. The ability of HSPGs to interact with ECM macromolecules such as collagen, laminin and fibronectin, and with different attachment sites on plasma membranes suggests a key role for these proteoglycans in the self-assembly and insolubility of ECM components, as well as in cell adhesion and locomotion. HSPGs are prominent components of blood vessels [9]. In large vessels they are mainly concentrated in the intima and inner media, whereas in capillaries they are found predominantly in the subendothelial basement membrane where they support proliferating and migrating endothelial cells, and stabilize the structure of the capillary wall. Cleavage of HS may therefore result in disassembly of the subendothelial ECM and hence may play a decisive role in extravasation of blood-borne cells.

Several cellular enzymes (i.e. collagenase IV, plasminogen activator, cathepsin B, elastase) are thought to be involved in degradation of basement membranes [10, 11]. Among these enzymes is an endo-β-D-glucuronidase (heparanase) that cleaves HS at specific intrachain sites [3, 4, 12]. The ability of cells to degrade HS in the ECM was studied by allowing cells to interact with a naturally produced sulfate-labeled ECM, followed by gel filtration (Sepharose 6B) analysis of degradation products released into the culture medium [3, 4]. Expression of a HS-degrading endoglucuronidase (heparanase) was found to correlate with the metastatic potential of mouse lymphoma [3], fibrosarcoma and melanoma [12], and with the ability of activated cells of the immune system to leave the circulation and elicit both inflammatory and autoimmune responses [13]. Moreover, elevated levels of heparanase were detected in serum from metastatic-tumor-

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bearing animals and melanoma patients [12] and in tumor biopsies of cancer patients [14].

Several studies have shown that heparanase activity expressed by either normal or neoplastic cells can be effectively inhibited by heparin, modified nonanticoagulant species of heparin and other sulfated polysaccharides [15–17]. Moreover, there was a reasonably good correlation between the heparanase-inhibiting activity of these compounds and their ability to inhibit tumor metastasis in experimental animals [16, 17]. Heparin is a multifunctional, linear, highly sulfated polysaccharide consisting of alternating uronic acid (either L-iduronic or D-glucuronic) and D-glucosamine residues. It exhibits a high degree of heterogeneity due to variation in the size of the polysaccharide chains and the degree and distribution of sulfate groups [18]. Although heparin is best known for its anticoagulant and antithrombotic properties, it affects a variety of physiological processes such as vascular endothelial and smooth muscle cell proliferation, angiogenesis, inflammation, autoimmunity and tumor metastasis [6, 7].

The present study was undertaken to determine the structural requirement (i.e. size, sulfate content and position) for inhibition of heparanase activity and tumor metastasis by species of heparin and heparin-like molecules. For this purpose, we investigated the ability of various chemically modified species of heparin and size-homogeneous oligosaccharides derived from depolymerized heparin to inhibit (1) heparanase-mediated degradation of HS in the subendothelial ECM, and (2) lung colonization of B16-BL6 melanoma cells in C57BL mice. Structural requirements determined in these experiments were compared to those identified in previous studies on the ability of these species of heparin to release ECM-bound basic fibroblast growth factor (b-FGF) [19] and promote its binding to cell surface receptor sites [20].

Materials and Methods

Materials

Sodium heparin from porcine intestinal mucosa (PM-heparin, M_r 14,000, anti-FXa 165 IU/mg, sulfur content 12%) was obtained from Hepar (Franklin, Ohio, USA). A low-M_r fragment (Kabi 2165, Fragmin) of this heparin (M_r 5,100, anti-FXa 130 IU/mg, sulfur content 12.4%) was prepared as a sodium salt by nitrous acid depolymerization [21]. Partially purified b-FGF was isolated from bovine brain, as described elsewhere [22]. Recombinant human b-FGF was kindly provided by Takeda (Osaka, Japan). Dulbecco's Eagle's medium (DMEM, 1 g glucose/l), calf serum, fetal calf serum (FCS), penicillin and streptomycin were obtained from Biological Industries (Beit-Haemek, Israel), as was saline containing 0.05% trypsin, 0.01 M sodium phosphate, and 0.02% EDTA (STV). Tissue culture dishes were from Falcon Labware Division, Becton Dickinson (Oxnard, Calif, USA). Four-well tissue culture plates were from Nunc (Roskilde, Denmark). $Na_2[^{35}S]O_4$ and $Na^{125}I$ were purchased from Amersham (Amersham, UK). Triton X-100, dextran T-40, and all other chemicals were of reagent grade, purchased from Sigma (St. Louis, Mo., USA).

Preparation and Characterization of Oligosaccharides from Nitrous-Acid-Depolymerized Heparins

Heparin fragments consisting of saccharide chains of different sizes were obtained by partial deaminative cleavage of heparin from porcine intestinal mucosa using nitrous acid, as described elsewhere [21]. Sizes ranged from disaccharides to chains with an average length of the starting heparin, i.e. 40–50 saccharides. Size-homogeneous oligosaccharides, similar to the starting heparin in their sulfur/carbon ratio, were also prepared by alkaline treatment of heparin methyl ester (β-elimination) as described elsewhere [23]. Similar results were obtained. The mixture of heparin fragments was subjected to ion exchange chromatography on DEAE Sepharose using sodium chloride as an eluent. Three fractions were collected: low-sulfate fraction, sulfur/carbon ratio (by elemental analysis) 0.14; medium-sulfate fraction, sulfur/carbon ratio 0.19, and high-sulfate fraction, sulfur/carbon ratio 0.21 [19, 23]. Each of the low-, medium- and high-sulfate fractions was further separated into size-homogeneous, even-numbered oligosaccharides by gel permeation chromatography on Sephadex G-50 Superfine [19, 23]. This procedure resulted in a set of oligosaccharides of different sizes and degrees of sulfation. The oligosaccharides with a higher sulfur/carbon ratio than heparin were found by

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^{1}H-NMR and ^{13}C-NMR spectroscopy to have their additional sulfate groups mainly in the glucosamines as indicated by a larger proportion of N-sulfate groups over N-acetyl groups and of 6-O-sulfate groups over 6-OH groups. The low-sulfate-containing oligosaccharides had a considerably reduced sulfate content in their glucosamines as well as in their iduronic acids [23].

Modified Heparins

Chemically modified nonanticoagulant species of heparin were prepared from native heparin and heparin fragment (Fragmin, M_r about 5,100). Briefly, the pyridinium salt of heparin and heparin fragment underwent complete N-desulfation by incubation with dimethyl sulfoxide and methanol [24]. Total desulfation of N and O sulfate groups was obtained by exhaustive desulfation with dimethyl sulfoxide containing 10% methanol and 0.4% trifluoroacetic acid. The N-desulfated heparin fragment was N-acetylated with acetic anhydride in water at pH 7–8 [19], or N-resulfated with sulfur trioxide trimethylamine complex, as described elsewhere [19]. An O-desulfated, N-acetylated heparin fragment was obtained by O-desulfating an N-acetylated heparin fragment, as described elsewhere [19, 25]. Intact heparin was chemically modified by the same procedures. These modified heparins exhibited <5% of the anticoagulant activity of heparin [15]. The chemical modifications made in the heparin fragments were assessed by ^{1}H-NMR and ^{13}C-NMR spectroscopy using a JEOL GX-400 instrument, 400 MHz for ^{1}H and 100 MHz for ^{13}C, and 2,2,3,3-tetradeuterio-3-trimethylsilylpropionate as an internal standard [23].

Cells

Cultures of bovine corneal endothelial cells were established from steer eyes as previously described [26]. Stock cultures were maintained in DMEM (1 g glucose/l) supplemented with 10% newborn calf serum, 5% FCS, 50 U/ml penicillin and 50 µg/ml streptomycin at 37°C in 10% CO_2-humidified incubators. Partially purified brain-derived b-FGF (100 ng/ml) was added every other day during the phase of active cell growth [5, 19]. Highly metastatic B16-BL6 melanoma cells, obtained by an in vitro selection procedure for invasion, were kindly provided by Dr. I.J. Fidler (University of Texas System Cancer Center, Houston, Tex., USA) [27]. Melanoma cells were maintained in culture in DMEM (4.5 g glucose/l) supplemented with 10% FCS, L-glutamine and antibiotics.

Preparation of Dishes Coated with ECM

Bovine corneal endothelial cells were dissociated from stock cultures (2nd–5th passage) with STV and plated into 4-well plates at an initial density of 2×10^5 cells/ml. Cells were maintained as described above except that 5% dextran T-40 was included in the growth medium and the cells were maintained without addition of b-FGF for 12 days. The subendothelial ECM was exposed by dissolving (5 min, room temperature) the cell layer with PBS containing 0.5% Triton X-100 and 20 mM NH$_4$OH, followed by four washes in PBS. The ECM remained intact, free of cellular debris and firmly attached to the entire area of the tissue culture dish [5, 19]. For preparation of sulfate-labeled ECM, corneal endothelial cells were plated into 4-well plates and cultured as described above. Na$_2$[^{35}S]O$_4$ (540–590 mCi/mmol) was added (40 µCi/ml) 2 and 5 days after seeding and the cultures were incubated with the label without medium change [3, 15]. Ten to twelve days after seeding, the cell monolayer was dissolved and the ECM exposed, as described above.

Degradation of Sulfated Proteoglycans

Sulfate-labeled ECM was incubated (3 h, 37°C, 10% CO_2 incubator) with intact cells or conditioned medium at pH 6.6 and 6.2, respectively. To evaluate the occurrence of proteoglycan degradation, the incubation medium was collected and applied for gel filtration on Sepharose 6B columns (0.9 × 30 cm). Fractions (0.2 ml) were eluted with PBS at a flow rate of 5 ml/h and counted for radioactivity using Bio-fluor scintillation fluid. The excluded volume (V_o) was marked by blue dextran and the total included volume (V_t) by phenol red. The latter was shown to comigrate with free sulfate [3, 15]. Degradation fragments of HS side chains were eluted from Sepharose 6B at $0.5 < K_{av} < 0.8$ (peak II) [3, 15]. A nearly intact HSPG released from ECM by trypsin – and, to a lesser extent, during incubation with PBS alone – was eluted next to V_o ($K_{av} < 0.2$, peak I). Recoveries of labeled material applied on the columns ranged from 85 to 95% in different experiments. Each experiment was performed at least three times and the variation of elution positions (K_{av} values) did not exceed ± 15%.

Tumor Metastasis

C57BL mice received a single subcutaneous (except when stated otherwise) injection of heparin (400 µg/ml/mouse, except when stated otherwise) 20 min prior to an intravenous inoculation of B16-BL6 melanoma cells (1×10^5 cells/mouse). Mice were sacrificed 15 days later, the lungs fixed in Bouen's solution and scored for the number of metastatic nodules [4, 16, 17, 28].
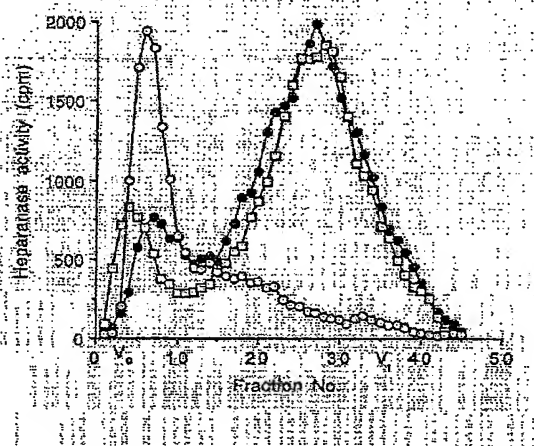
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Fig. 1. Degradation of sulfate-labeled ECM by B16-BL6 melanoma cells. B16-BL6 melanoma cells (1×10^6/35-mm dish) were incubated (24 h, 37°C, pH 6.6) in contact with sulfate-labeled ECM in the absence (□) and presence (○) of 5 µg/ml heparin, or 20 µg/ml N/O-desulfated heparin (●). Sulfate-labeled degradation products released into the incubation medium were analyzed by gel filtration over Sepharose 6B.
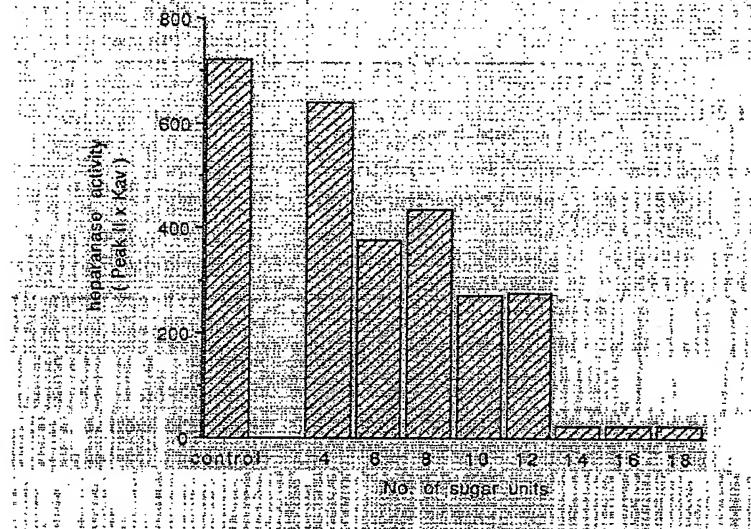
Results

Effect of Oligosaccharides Derived from Depolymerized Heparin on Heparanase Activity

Sulfate-labeled ECM was incubated (24 h, 37°C) with melanoma cells (fig. 1) or melanoma-cell-conditioned medium (fig. 2) in the absence and presence of intact heparin, totally desulfated heparin (fig. 1) or size-homogeneous oligosaccharides prepared from heparin by nitrous acid depolymerization (fig. 2). Sulfate-labeled material released into the incubation medium was analyzed by gel filtration on Sepharose 6B. In the presence of the incubation medium alone, there was a constant release of labeled material that consisted almost entirely (>90%) of large-M_r fragments eluted with or next to V_o. We have previously

shown that a proteolytic activity residing in the ECM itself [4] and/or expressed by cells is responsible for the release of the high-M_r material. This nearly intact HSPG provides a soluble substrate for subsequent degradation by heparanase, as also indicated by the relatively large amount of peak I material accumulating when the heparanase enzyme was inhibited by heparin [15]. On the other hand, incubation of the labeled ECM with B16-BL6 melanoma cells resulted in the release of 60–70% of the ECM-associated radioactivity in the form of low-M_r sulfate-labeled fragments (peak II, $0.5 < K_{av} < 0.75$) (fig. 1). Degradation fragments eluted in peak II were shown to be degradation products of HS, as they were (1) 5- to 6-fold smaller than intact HS side chains (K_{av} about 0.33) released from ECM by treatment with either alkaline borohydride or papain, and (2) resistant to further digestion with papain or chondroitinase ABC and susceptible to deamination by nitrous acid [3, 18]. Accumulation in the medium of low-M_r sulfate-labeled degradation fragments was inhibited by heparin, a potent inhibitor of heparanase-mediated HS degradation [15], but not by N/O-desulfated heparin (fig. 1). In subsequent experiments, heparanase activity is expressed as the total amount of radioactivity eluted in peak II (fractions 25–35, $0.5 < K_{av} < 0.75$) multiplied by the K_{av} of peak II, thereby taking into account both the total amount and size of the HS degradation fragments. As demonstrated in figure 2, complete inhibition of heparanase was observed in the presence of 10 µg/ml of the tetradecasaccharide. Similar results were obtained with oligosaccharides containing 16 and 18 sugar units, while smaller oligosaccharides yielded only a partial inhibition of heparanase even at a concentration of 25 µg/ml. Similar results were obtained with oligosaccharides derived from heparin by nitrous acid depolymerization (fig. 2) or by alkaline β-elimination (not shown).

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Fig. 2. Inhibition of heparanase by heparin-derived oligosaccharides of varying sizes. Sulfate-labeled ECM-coated 35-mm dishes were incubated (37°C, 24 h, pH 6.2) with melanoma cell heparanase (serum-free medium conditioned by mouse B16-BL6 melanoma cells) in the absence (control) and presence of 10 µg/ml size-homogeneous oligosaccharides (4–18 sugar units) prepared by nitrous acid depolymerization of heparin. Sulfate-labeled material released into the incubation medium was analyzed by gel filtration over Sepharose 6B columns. Heparanase activity is expressed by the amount of radioactivity eluted in peak II (fractions No. 25–35) multiplied by the K_{av} of this peak $(0.5 < K_{av} < 0.8,$ M_r about 4,000–8,000).

We next compared the effect of oligosaccharides derived from nitrous-acid-degraded heparin and having varying degrees of sulfation. A high-sulfate fraction (%sulfate = 12.5) of the tetradecasaccharide was a 7- and 5-fold better heparanase inhibitor than low (%sulfate = 6.6) and moderately (%sulfate = 9.3) sulfated fractions of the same oligosaccharide, respectively (fig. 3).

Effect of Chemically Modified Species of Heparin on Heparanase Activity

As demonstrated in figure 4, there was no inhibition of heparanase activity by either totally N/O-desulfated (%sulfate <1), N-desulfated (%sulfate = 9.7), or N/O-desulfated, N-resulfated (%sulfate = 5.3) species of heparin, indicating that both the N- and O-sulfates of heparin are required for inhibition of heparanase. Substitution of the N-sulfates of heparin with acetyl or hexanoyl groups (%sulfate = 8.7 and 7.5, respectively) yielded species of heparin which inhibited heparanase activity nearly as well as native heparin or heparin
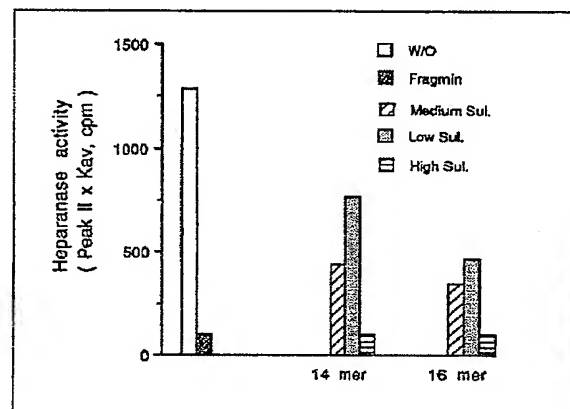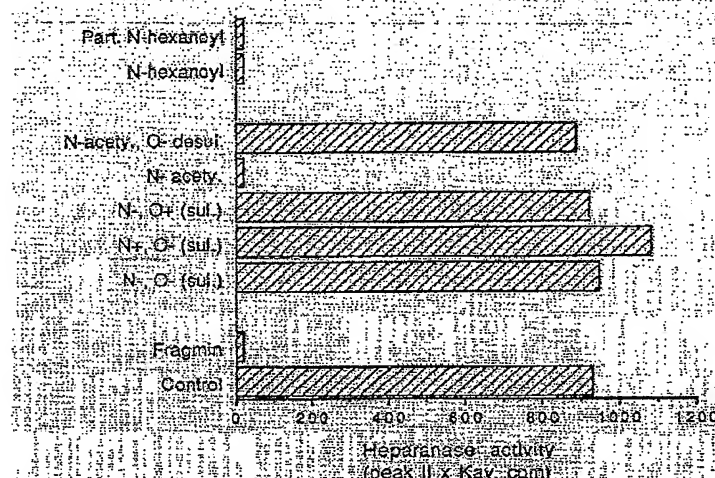

Fig. 3. Inhibition of heparanase by heparin-derived oligosaccharides with low, medium and high sulfate content. Sulfate-labeled ECM was incubated (37°C, 24 h, pH 6.2) with melanoma cell heparanase in the absence (W/O) and presence of 2 µg/ml fragmin or 2 µg/ml of low-, medium- (normal) and high-sulfate fractions of a tetradecasaccharide and hexadecasaccharide obtained from heparin by nitrous acid depolymerization and ion exchange chromatography on DEAE Sepharose, as described in Materials and Methods. The incubation medium was subjected to gel filtration on Sepharose 6B and heparanase activity is expressed as described in the legend to figure 2.

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Fig. 4. Inhibition of heparanase by chemically modified species of heparin fragment. Sulfate-labeled ECM was incubated (37°C, 24 h, pH 6.2) with melanoma cell heparanase in the absence (control) or presence of 2.5 µg/ml heparin fragment (Fragmin), totally desulfated (N–, O–) heparin fragment and species of heparin fragment chemically modified to contain sulfate groups at either the N-position (N+, O–) or O-position (N–, O+). N-sulfates were also partially or totally substituted with acetyl (N-acetyl) or hexanoyl (N-hexanoyl) groups, or by acetyl groups followed by O-desulfation. Sulfate-labeled material released into the incubation medium was analyzed by gel filtration over Sepharose 6B and heparanase activity is expressed as described in the legend to figure 2.
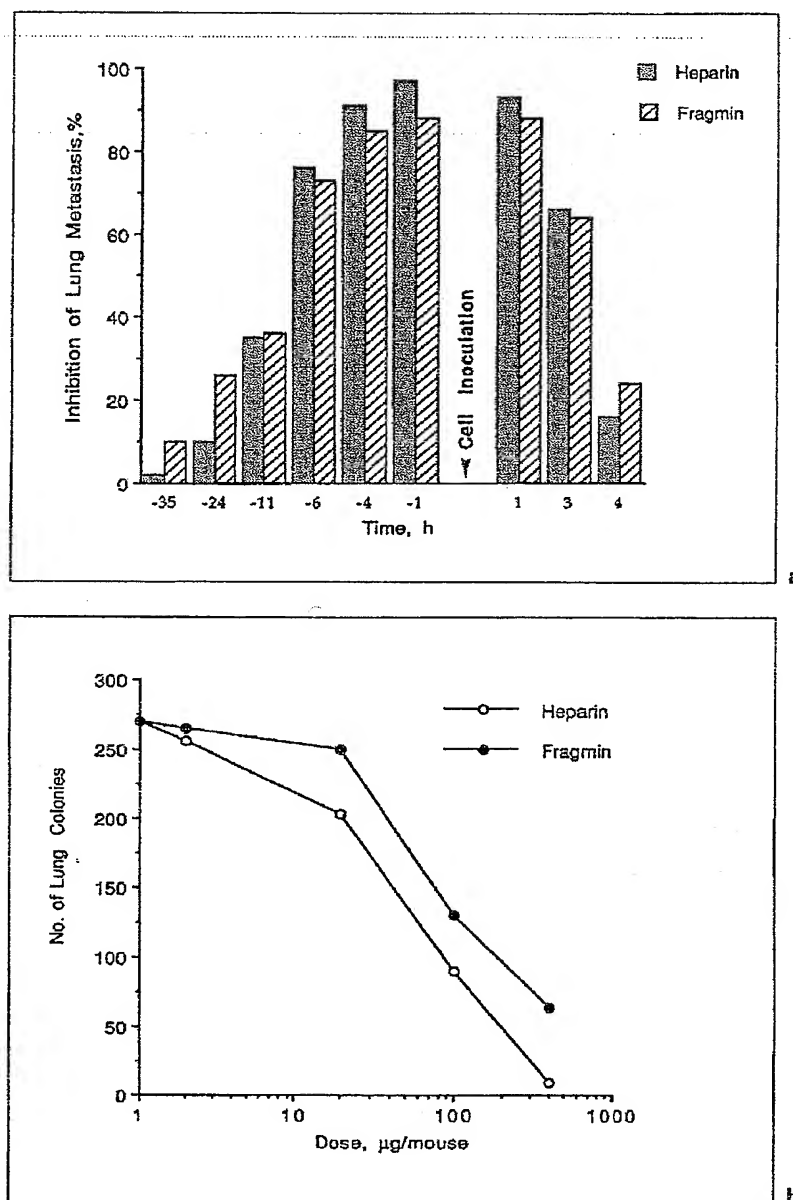

fragment (fig. 4). Heparin fractions with high and low affinity to antithrombin III exhibited a similar high heparanase inhibitory activity, despite a 200-fold difference in their anticoagulant activity (not shown).

Inhibition of Tumor Metastasis by Species of Heparin

Treatment of tumor cells and animals with heparanase-inhibiting species of heparin markedly reduced the incidence of lung metastases induced by B16 melanoma [16], Lewis lung carcinoma [28] and mammary adenocarcinoma cells [17]. A single injection of heparin or heparin fragment (400 µg/mouse) given 1 h before or 1 h after intravenous inoculation of B16-BL6 melanoma cells (10^5 cells/mouse), decreased the number of melanoma lung metastases to about 5% of control values (fig. 5a). About 65 and 75% inhibition of metastasis was achieved when heparin was given 3 h after or 6 h before the

tumor cells, respectively, but there was little or no inhibition when the tumor cells were inoculated 4 h prior to the heparin treatment (fig. 5a). These results indicate that the polysaccharide interferes with the passage of tumor cells across the capillary wall [17]. Similar results were obtained regardless of whether the heparin was injected intravenously, subcutaneously or intraperitoneally. Efficient inhibition of experimental B16 melanoma lung colonization was obtained at heparin concentrations (100–400 µg/mouse) (fig. 5b) much higher than those which inhibited the heparanase enzyme in vitro (2.5–10 µg/ml). Heparin fractions with high and low affinity for antithrombin III exhibited comparably high antimetastatic activities (fig. 6, 7), indicating that the heparanase-inhibiting activity of heparin rather than its anticoagulant activity plays a role in the antimetastatic properties of the polysaccharide. The most efficient inhibition of tumor cell metastasis was obtained when

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Fig. 5. Effect of heparin and Fragmin on lung colonization of B16-BL6 melanoma cells. C57BL mice received a single subcutaneous injection of Fragmin or heparin (400 µg/mouse) and an intravenous inoculation of B16-BL6 melanoma cells (10^5 cells/mouse). Mice were sacrified 15 days later, the lungs fixed in Bouen's solution and scored for the number of metastatic nodules. **a** Percent inhibition of lung colonization by heparin and Fragmin injected (subcutaneous) at various times before (−) or after (+) inoculation of B16-BL6 cells into the tail vein of C57BL mice. **b** Dose response. C57BL mice received a single subcutaneous injection of heparin or Fragmin 5–10 min prior to an intravenous inoculation of B16-BL6 melanoma cells (10^5 cells/mouse). Each data point represents the mean of lung nodules in 5–6 mice and the variation between different animals did not exceed ±25% of the mean.
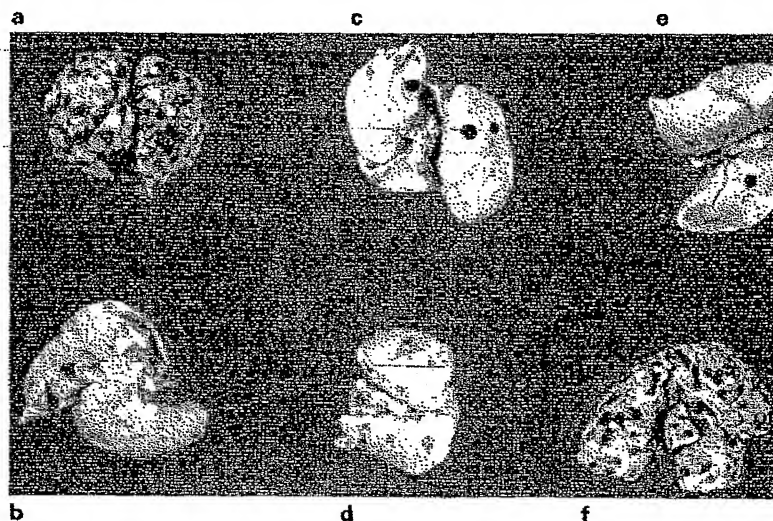
the melanoma cells and heparin were injected at the same time.

Lung colonization of B16 melanoma cells was efficiently inhibited by N-hexanoyl heparin or heparin fragment, but not by N-desulfated, or N/O-desulfated, N-resulfated, or totally N/O-desulfated heparin fragment (fig. 6, 7), in correlation with the heparanase-inhibiting activity of these species of heparin. Tumor metastasis was inhibited to a greater

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Fig. 6. Effect of heparin species on lung colonization of B16-BL6 melanoma cells. C57BL mice received a single subcutaneous injection of saline (control) (a), or 400 μg/ml of heparin fragment (b), N-hexanoyl heparin fragment (c), Fragmin with high (d) and low (e) affinity to antithrombin III, or totally desulfated fragmin (f), followed by an intravenous inoculation of B16-BL6 cells (10^5 cells/mouse). Fifteen days afterwards, the mice were sacrified and the lungs fixed in Bouin's solution.

extent by N-hexanoyl as compared to N-acetylated heparin fragment. N-acetylated heparin was more active than N-acetylated heparin fragment (fig. 7). Oversulfated, N-acetylated heparin fragment (%sulfate = 13.9) was a more efficient inhibitor of lung colonization (>90% inhibition) than partially or totally N-acetylated heparin fragment. Treatment with the hexadecasaccharide yielded >90% inhibition of lung colonization, but there was only a small inhibition by heparin-derived oligosaccharides containing 12 sugar units or less (not shown).

Discussion

A number of studies have shown that sulfated polysaccharides (i.e. heparin, dextran sulfate, pentosan polysulfate, xylose sulfate) can inhibit tumor growth and metastasis [29–31]. Although all of the inhibitory polysaccharides were anticoagulants, the antimetastatic potential of the molecules did not correlate with their anticoagulant activity [17, 29]. There was also no correlation between the sul-

fated polysaccharides that bound to the surface of tumor cells and those that inhibited metastasis [29]. Moreover, inhibitory polysaccharides did not affect adhesion of tumor cells to the vascular endothelium [29]. The inhibition of tumor growth by pentosan polysulfate [31] and other polyanionic molecules [32] was also attributed to interaction with heparin-binding growth factors (i.e. b-FGF) which were no longer capable of promoting autocrine cell proliferation. Parish et al. [17] have demonstrated that sulfated polysaccharides inhibit metastatic dissemination of rat mammary adenocarcinoma cells by inhibiting tumor-cell-derived heparanase involved in the penetration of the vascular endothelium and its underlying basement membrane by tumor cells [17]. This conclusion was strengthened in the present study by applying both chemically modified species of heparin and size-homogeneous oligosaccharides derived from depolymerized heparin. Those species of heparin that inhibited heparanase-mediated degradation of heparan sulfate in intact ECM were also potent inhibitors of B16 melanoma extravasation and lung colonization. The
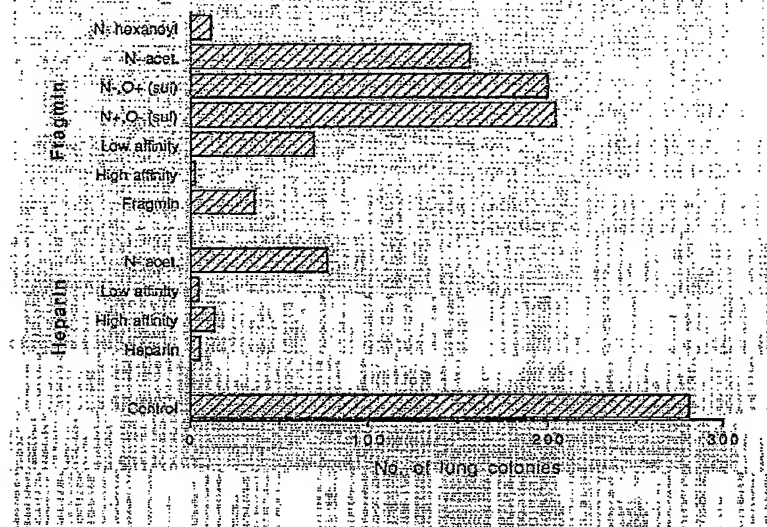
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Fig. 7. Inhibition of tumor metastasis by chemically modified species of heparin. C57BL mice received a single intravenous injection (400 µg/mouse) of native and chemically modified species of heparin and heparin fragment followed by an intravenous inoculation of B16-BL6 cells (10^5 cells/mouse). Two weeks afterwards, the mice were sacrificed and the lungs scored for the number of melanoma nodules. The following compounds were tested: heparin; N-acetylated heparin; heparin fragment (Fragmin); N-acetylated heparin fragment; N/O-desulfated, N-resulfated fragmin; N-desulfated fragmin, N-hexanoyl fragmin, and heparin or fragmin with high and low affinity for antithrombin III. Each data point is the mean of the number of lung colonies in 5–6 mice. The variation between different animals did not exceed 25%.

availability of a large collection of chemically modified and low-M_r species of heparin enabled us to determine the structural requirements for inhibition of heparanase activity and tumor metastasis, in comparison with other heparin-mediated effects such as release of ECM-bound b-FGF [19] and stimulation of b-FGF binding to high-affinity cell surface receptors [20, 33].

Although heparin is best known for its anticoagulant and antithrombotic properties, it affects various physiological processes such as vascular endothelial and smooth muscle cell proliferation, angiogenesis, inflammation and autoimmunity [9, 13]. It is, however, virtually impossible to assign structure-function relationships based on studies with native heparin due to variations in the size of the polysaccharide chains and in the degree and distribution of sulfate groups [18, 23]. To elucidate the structural requirements for inhibition of heparanase and tumor metastasis by heparin, we have used a number of chemically modified species of heparin fragment and homogeneously sized oligosaccharides obtained from depolymerized heparin. Inhibition of heparanase and tumor metastasis was achieved by heparin species containing 16 sugar units or more and having sulfate groups at both the N and O positions. Low-sulfate oligosaccharides were less effective heparanase inhibitors than medium- and high-sulfate

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fractions of the same size. While O-desulfation abolished the heparanase-inhibiting effect of heparin, N-acetylated or N-hexonyl heparin retained a high inhibitory activity, provided that the N-substituted molecules had a molecular size ≥4,000 D. Efficient inhibition of melanoma lung colonization by N-acetylated heparin was also reported by Irimura et al. [16]. In contrast to these results, we have previously demonstrated that the presence of N-sulfates is a critical requirement for release of ECM-bound b-FGF by heparin species [19]. It was found that total substitution of N-sulfates with acetyl or hexanoyl groups resulted in an almost complete inhibition of the b-FGF-releasing activity of heparin, despite a normal content of O-sulfate groups [19]. O-sulfation facilitated b-FGF release, but was not an absolute requirement, as indicated by the relatively high b-FGF-releasing activity of totally N/O-desulfated, N-resulfated heparin fragment [19]. Unlike the inhibition of heparanase activity and lung colonization, a nearly maximal release of ECM-bound b-FGF was obtained by the octasaccharide, while the tetrasaccharide exhibited about 40% of the activity of intact heparin when compared on a weight basis [19]. No correlation was found between the antithrombotic activity (antifactor Xa activity) of heparin and its antimetastatic and b-FGF-releasing activities, indicating that the specific pentasaccharide sequence responsible for the binding of anticoagulant heparin to antithrombin III is not required for inhibition of heparanase and release of ECM-bound b-FGF.

Heparin or HSPGs are involved in the receptor binding and mitogenic activity of b-FGF [33, 34]. We have recently investigated the capacity of various species of heparin and HS to promote b-FGF receptor binding using both CHO mutant cells deficient in cell surface HSPG and a soluble b-FGF receptor-alkaline phosphatase fusion protein [20].

b-FGF receptor binding was induced by heparin-derived oligosaccharides containing as little as 8–10 sugar units [20, 33]. High-sulfate oligosaccharides were more effective than medium- and low-sulfate fractions of the same-size oligosaccharide. The highest level of b-FGF receptor binding was achieved in the presence of oversulfated heparin fragments, regardless of whether the N position was sulfated or acetylated [20]. Thus, there was an absolute requirement for O-sulfation and a synergistic effect of N-linked sulfates. These structural characteristics of heparin are distinctly different from those sufficient for displacement of b-FGF bound to HS on cell surfaces and ECM [19]. While a significant displacement of HS-bound b-FGF was obtained by heparin fragments containing as little as 4–6 sugar units and by N-sulfated, O-desulfated heparin fragments [19], these species of heparin failed to promote high-affinity b-FGF receptor binding and activation [20, 33]. As discussed above, there was also no correlation between the ability of various modified and low-molecular-weight species of heparin to release b-FGF from ECM and their capacity to inhibit the enzyme heparanase and melanoma cell metastasis. These results further indicate that different effects of heparin are mediated by different sugar sequences and that unique heparin-like molecules can be designed to elicit or inhibit a specific function. For example, N-substituted species of heparin and in particular N-hexanoyl heparin fragment, rather than native heparin, could be applied to inhibit tumor metastasis, since their efficient inhibition of heparanase activity was not associated with a significant release of active b-FGF from cells and ECM. These compounds are therefore expected to inhibit metastases by certain tumor cells, correlated with their inhibition of heparanase activity, with little or no potential induction of tumor angiogenesis in response to b-FGF release. On

the other hand, oligosaccharides derived from depolymerized heparin and containing 8–10 sugar units, or N-sulfated, O-desulfated species of heparin can possibly be applied to stimulate neovascularization and wound healing by virtue of their efficient b-FGF-releasing activity. These molecules are not expected to interfere, for example, with extravasation of blood-borne cells and hence with the normal function of the immune system since they do not inhibit the heparanase enzyme expressed by activated lymphocytes, neutrophils and mast cells [13, 35].

Acknowledgments

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